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ISOLATION OF LPL GENE FROM HUMAN BLOOD SAMPLE

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ABSTRACT

Isolation of DNA from living cells is very important in molecular biological studies. You may have heard about DNA finger printing, genetic engineering…etc; all these molecular biological works in need of isolated pure DNA.

Lipoprotein Lipase (LPL): (EC3.1.1.34) Is a member of the lipase gene family, which includes pancreatic lipase, hepatic lipase, and endothelial lipase. It is a water soluble enzyme that hydrolyzestriglycerides in lipoproteins, such as those found in chylomicrons and very low-density lipoproteins (VLDL), into two free fatty acids and one monoacylglycerol molecule. It is also involved in promoting the cellular uptake of chylomicron remnants, cholesterol-rich lipoproteins, and free fatty acids. LPL requires Apo-CII as a cofactor. LPL is attached to the luminal surface of endothelial cells in capillaries. It is most widely distributed in adipose, heart, and skeletal muscle tissue, as well as in lactating mammary glands.

KEYWORDS: Isolation of LPL Gene From Human Blood Sample, Apo-CII, Fatty Acids and One Monoacylglycerol Molecule

INTRODUCTION

Synthesis: In brief, LPL is secreted from parenchymal cells as a glycosylatedhomodimer, after which it is translocated through the extracellular matrix and across endothelial cells to the capillary lumen. After translation, the newly synthesized protein is glycosylated in the endoplasmic reticulum. The glycosylation sites of LPL are Asn-43, Asn-257, and Asn-359.[1]Glucosidases then remove terminal glucose residues; it is believed that this glucose trimming is responsible for the conformational change needed for LPL to form homodimers and become catalytically active. In the Golgi apparatus, the oligosaccharides are further altered to result in either two complex chains, or two complex and one high-mannose chain. In the final protein, carbohydrates account for about 12% of the molecular mass. Homodimerization is required before LPL can be secreted from cells.Aftersecretion, however, the mechanism by which LPL travels across endothelial cells is still unknown.

Structure: The crystal structure of LPL has not been discovered; however, there are substantial experimental evidence and structural homology between members of the lipase family to predict the likely structure and functional regions of the enzyme. LPL is composed of two distinct regions: the larger N-terminus domain that contains the lipolyticactive site, and the smaller C-terminus domain. These two regions are attached by a peptide linker. The N-terminus domain has anα/β hydrolase fold, which is a globular structure containing a central β sheet surrounded by α helices. The C-terminus domain is a β sandwich formed by two β sheet layers, and resembles an elongated cylinder.

Functions: LPL encodes lipoprotein lipase, which is expressed on endothelial cells in the heart, muscle, and adipose tissue. LPL functions as a homodimer, and has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake.

Through catalysis, VLDL is converted to IDL and then to LDL. Severe mutations that cause LPL deficiency result in type I hyperlipoproteinemia, while less extreme mutations in LPL are linked to many disorders of lipoprotein metabolism.

LITERATURE REVIEW

Lipoprotein lipase is an enzyme which hydrolyses the TAGs into monoacylglycerols and fatty acids. The gene encoding the LPL is located on the 8th chromosome of human genome. The mutation in this particular gene or even the presence or absence of the gene is a deciding factor for certain disorders like diabetes, hypertension and obesity.

List of the literature obtained on the LPL gene based on the review papers:

Protein Coding Potential: 6 spliced and the unspliced mRNAs putatively encode good proteins, altogether 7 different isoforms (4 complete, 1 COOH complete, 2 partial), some containing domains Lipase, PLAT/LH2 domain; 2 of the 4 complete proteins appear to be secreted. The remaining 4 mRNA variants (1 spliced, 3 unspliced; 2 partial) appear not to encode good proteins.

- **Expression:** According to AceView, this gene is expressed at very high level, 4.7 times the average gene in this release. The sequence of this gene is defined by 548 GenBank accessions from 495 cDNA clones, some from brain (seen 126 times), lung (51), carcinoid (29), parathyroid gland (27), parathyroid tumour (27), heart (25), cerebellum (22) and 80 other tissues. We annotate structural defects or features in 12 cDNA clones.
- **Map:** This gene LPL maps on chromosome 8, at 8p22 according to Entrez Gene. In AceView, it covers 28.50 kb, from 19796284 to 19824778 (NCBI 37, August 2010), on the direct strand.
- **Links to:** manual annotations from OMIM_144250, OMIM_238600, GAD, KEGG_00561, KEGG_03320, KEGG_05010, the SNP view gene overviews from Entrez Gene 4023, GeneCards, expression data from ECgene, UniGene, molecular and other annotations fromUCSC, or our GOLD analysis.
- **Lipoprotein lipase (LPL)**: deficiency is an autosomal recessive disease for which there is no drug therapy available, and is associated with severe hypertriglyceridemia, severe chylomicronemia, and low high-density lipoprotein levels, which often leads to acute pancreatitis.

METHODOLOGY

ISOLATION OF GENOMIC DNA FROM HUMAN LYMPHOCYTES (TRITON-X 100 METHOD)

REQUIREMENTS

- Human blood
- Glass and plastic ware & others: Micropipettes, tips, microfuge tubes etc.
- Instruments: Cooling centrifuge
- Chemicals:
    - Lysis Buffer(0.32 M sucrose, 1% Triton X-100, 1 mM $MgCl_2$, 12 mMTris-HCL(ph 8.0))
    - 20% SDS

- Proteinase K Buffer (0.375 M NaCl, 0.12 M EDTA, Proteinase K (1mg/ml) (ph 8.0))
- Phenol:Chloroform(1:1)
- Ethanol(absolute and 70%)
- TE Buffer (10mM TrisHCl, 1mM EDTA(ph 8.0).

PROCEDURE

- Take 1 ml of blood and add 1 ml lysis buffer. Mix well and centrifuge at 10000 rpm for 10 min at 4^{o}C.
- Discard the supernatant and resuspend the pellet in 400 µl of lysis buffer and centrifuge at 10000 rpm for 5 min at 4^{o}C and repeat the process once again
- Discard the supernatant and resuspend the pellet in 400 µl of distilled water. Centrifuge at 10000 rpm for 5 min at 4^{o}C.
- Discard the supernatant and add 100 µl of Proteinase K buffer and 10 µl 20% SDS. Resuspend the pellet and mix it till frothing.
- Add 140 µl NaCl and mix well. Add 400 µl distilled water and 400 µl Phenol: Chloroform.
- Mix well and centrifuge at 10000 rpm for 10 min at 4^{o}C to separate the viscous and aqueous phase.
- Transfer the aqueous phase to fresh tube and add 1 ml chilled ethanol to precipitate DNA. Leave at -20^{o}C for 1-2 hr or longer.
- Spin at 10000 rpm for 20 min at 4^{o}C. Pour off the supernatant and wash the pellet with 70% ethanol. Spin 10000 rpm for 5 min at 4^{o}C, pour off ethanol, air dry the pellet and dissolve in about 50 µl TE.
- The technique was standardized using normal blood samples. On obtaining good results, this technique was later extended for DNA isolation from diseased blood samples.

SPECTROPHOTOMETRIC METHOD FOR QUANTIFICATION OF DNA

The purity and the concentration of genomic DNA were checked using spectrophotometer at $OD_{260/280}$ taken against TE as a blank. The DNA sample showing the OD 260/280 value between 1.7-1.9 was considered as pure sample and the concentration of genomic DNA was estimated as follows:

DNA concentration (µg/µl) = (OD260 *Dilution Factor* 50)/ 1000

PRIMER DESIGN USING PRIMER 3 TOOL

Primer sequences need to be chosen to uniquely select for a region of DNA, avoiding the possibility of mishybridization to a similar sequence nearby. A commonly used method is BLAST search whereby all the possible regions to which a primer may bind can be seen. Both the nucleotide sequence as well as the primer itself can be BLAST searched. The free NCBI tool Primer-BLAST integrates primer design tool and BLAST search into one application,[4] so does commercial software product such as ePrime, Beacon Designer. Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design.[15] Mononucleotide repeats should be avoided, as loop

formation can occur and contribute to mishybridization. Primers should not easily anneal with other primers in the mixture (either other copies of same or the reverse direction primer); this phenomenon can lead to the production of 'primer dimer' products contaminating the mixture. Primers should also not anneal strongly to themselves, as internal hairpins and loops could hinder the annealing with the template DNA. Pairs of primers should have similar melting temperatures since annealing in a PCR occurs for both simultaneously. A primer with a T_m significantly higher than the reaction's annealing temperature may mishybridize and extend at an incorrect location along the DNA sequence, while T_m significantly lower than the annealing temperature may fail to anneal and extend at all. When designing a primer for use in TA cloning, efficiency can be increased by adding AG tails to the 5' and the 3' end. The reverse primer has to be the reverse complement of the given cDNA sequence. The reverse complement can be easily determined, e.g. with on-line calculators.

PCR PROTOCOL

The **Polymerase Chain Reaction** (**PCR**) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size.[5]The reaction produces a limited amount of final amplified product that is governed by the available reagents in the reaction and the feedback-inhibition of the reaction products.[6]

A basic PCR set up requires several components and reagents.[7] These components include:

- *DNA template* that contains the DNA region (target) to be amplified.
- Two *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70 °C.
- *Deoxynucleoside triphosphates* (dNTPs; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- *Buffer solution*, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- *Divalentcations*, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis[8]
- *Monovalent cation*potassium ions.

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction (see below). Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the

reaction mixture or a ball of wax inside the tube.

Procedure

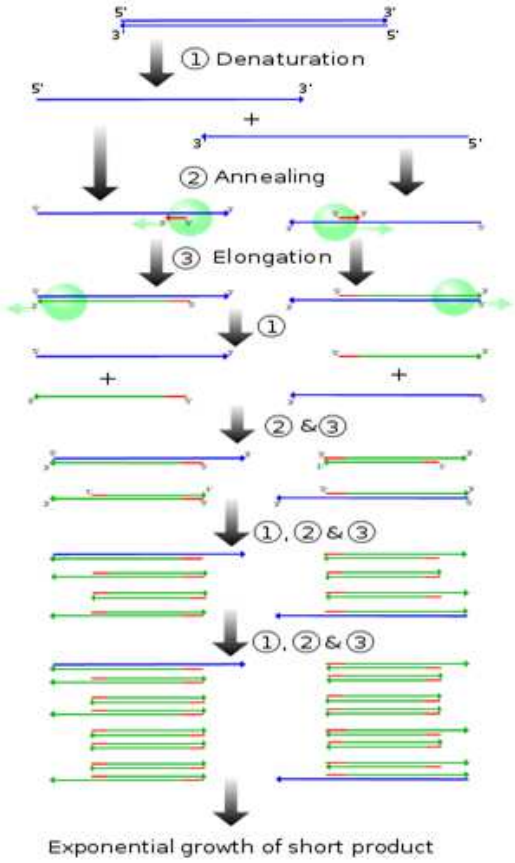

Figure 1

Figure: Schematic drawing of the PCR cycle. (1) Denaturing at 94–96 °C. (2) Annealing at ~65 °C (3) Elongation at 72 °C. Four cycles are shown here. The blue lines represent the DNA template to which primers (red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (green lines), which themselves are used as templates as PCR progresses.

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three (Figure 3). The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.[9]

- **Initialization Step:** This step consists of heating the reaction to a temperature of 96–98 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.[10]
- **Denaturation Step:** This step is the first regular cycling event and consists of heating the reaction to 96–98 °C

for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

- **Annealing Step:** The reaction temperature is lowered to 55–60 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
- **Extension/Elongation Step:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 68-70°C,[11][12] and commonly a temperature of 68°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.
- **Final Elongation:** This single step is occasionally performed at a temperature of 68-70 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Gel Documentation

Gel Preparation

Reagents Required

- Agarose
- Ethedium bromide
- 1X TAE buffer

Preparation of 0.6% Agarose: 0.3g of agarose in 50 ml of 1X TAE buffer. Add 2µl of EtBr. Heat to dissolve the agarose using magnetic stirrer. Pour the preparation in the gel electrophoretic unit. Let it to cool. Remove off the comb which leaves behind 8 wells which is further loaded with the stained DNA sample.

Gel Electrophoresis

Load all the 8 wells with marker as well as the PCR products. Connect the electrodes, run the gel at 50V for about 2 hours. Analysis of bands obtained can be done by using gel doc instrument using UV.

RESULTS

Spectrometri Results

Tabulation

Table 1

Protocol Used	Absorbance at 260nm	Absorbance at 280nm	Purity	DNA Concentration µg/µl
Treated with Ethanol	0.520	0.607	0.8567	0.4284

Purity of DNA when ethanol is used = A_{260}/A_{280}

=0.520/0.607

=0.8567

DNA concentration for ethanol (µg/µl)= (OD at 260nm*dilution factor*50)/ 1000

= (0.520*10*50)/1000

= 0.4284 µg/µl

Quantification Using UV Spectrophotometer

Tabulation

Table 2

Protocol Used	Absorbance at 260nm	Absorbance at 280nm	Purity	DNA Concentration µg/µl
Treated with Ethanol	0.293	0.123	1.4	0.1319
Treated with Isopropyl alcohol	0.213	0.074	1.667	0.0554

Purity of DNA when ethanol is used = A_{260}/A_{280}

=0.293/0.123

=1.4

Purity of DNA when isopropyl alcohol is used = A_{260}/A_{280}

=0.213/0.074

=1.667

DNA concentration for ethanol (µg/µl)= (OD at 260nm*dilution factor*50)/ 1000

= (0.293*9*50)/1000

= 0.1319 µg/µl

DNA concentration for isopropyl alcohol (µg/µl)= (OD at 260nm *dilution factor*50)/ 1000

= (0.213*9*50)/1000

= 0.0554 µg/µl

After analyzing the DNA purity and concentrations, we found that the sample treated with ethanol has purity 1.4 and DNA concentration 0.1319 µg/µl and that treated with isopropyl alcohol has purity 1.667 and concentration 0.0554 µg/µl. The standard DNA purity must have to lie in the range of 1.2-1.8. As we have found the purity of both the samples lying within the range, moreover comparing the DNA concentrations, the sample treated with ethanol is found to have higher concentration than the other sample. Hence we choose the sample treated with ethanol for further PCR applications.

PCR Results

The sample of DNA obtained from human blood was subjected to PCR using suitable forward and reverse primers.

The targeted exon is- exon 9 whose sequence is:

Exon 9*: F: GTTCTACATGGCATATTCAC

R: TAGCCCAGAATGCTCACCAGACT

The expected result must include the amplification of the LPL gene which can be further used for gel documentation.

Gel Electrophoresis Results

The samples isolated from the mutated blood and the normal blood were subjected to gel electrophoresis and the results are as shown below:

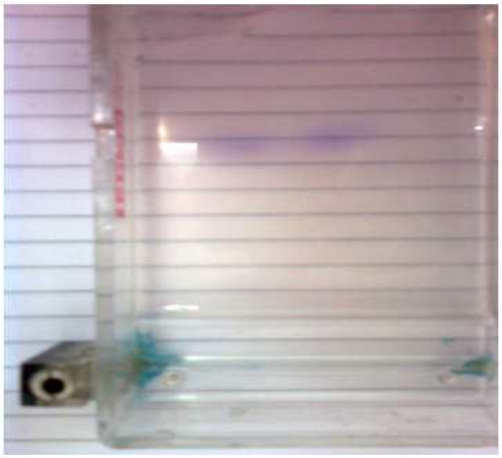

Figure 2

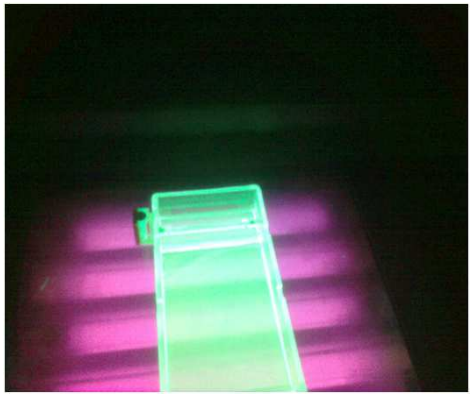

Figure 3

Figure 4

Figure 5

CONCLUSIONS

LPL is a lipolytic enzyme and is essential for the hydrolysis of CM & VLDL triglycerides and a functional defect of LPL causes marked hyperchylomicronemia or hyper triglyceredemia. To date more than 100 mutations have been identified in the LPL gene around the world, and the mutation in the LPL gene have been linked to several disease such as FCHL, premature artherosclerosis, Alzheimer's disease, hyper tension etc. but the frequency of individual LPL mutations

differs widely between regions or populations. In this study the coding regions and exon-intron junctions of the LPL gene was examined with or without the use of HTG, one miss-sense mutation P207L, three splicing mutations Int3/3'-ass/C(-6)--T, one novel silent mutation L103L and the common S447X polymorphism has been identified. It can be used for further mutational analysis and the multiple alleles associated with the particular defectives also we can identify the novel mutations and strive for the development of Insilco drug to target the disease and majorly helps in the molecular study of the gene and its associated mutagenic effects through clinical trials.

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